S100A7 (Psoriasin) Interacts with Epidermal Fatty Acid Binding Protein and Localizes in Focal Adhesion-Like Structures in Cultured Keratinocytes

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S100 proteins are calcium-responsive signaling proteins that are overexpressed in cancer and inflammatory diseases. They act by forming complexes with target proteins to modify target protein function. Identifying S100 intracellular distribution, site of action, and protein targets are important goals. S100A7 (psoriasin) is an important member of this family that is markedly overexpressed in psoriatic keratinocytes; however, its role in disease progression is poorly understood. In this study, we express S100A7 in normal keratinocytes as a means to study S100A7 function. We show that S100A7 is present in the cytosol and in BiP/GRP78-positive (endoplasmic reticulum) tubular structures. When cells are challenged with elevated intracellular calcium, cytoplasmic S100A7 redistributes to α-actinin- and paxillin-positive peripheral structures that contact the substrate surface. Epidermal fatty acid binding protein is also overexpressed in psoriasis, and is a putative target of S100A7 in keratinocytes. To study this interaction, we coexpressed S100A7 and epidermal fatty acid binding protein. These studies indicate that S100A7 and epidermal fatty acid binding protein colocalize in the cytoplasm in untreated cultures, and localize in peripheral structures in response to calcium challenge. In addition, S100A7 expression appears to stabilize epidermal fatty acid binding protein level, and vice versa. Moreover, the proteins can be coprecipitated in the presence of bifunctional cross-linker, suggesting that they are part of a common complex. The colocalization with α-actinin and paxillin suggests that S100A7 and epidermal fatty acid binding protein colocalize in focal adhesion-like structures following calcium treatment. ***Key words: epidermal fatty acid binding protein/keratinocyte differentiation/psoriasin/psoriasis/signal transduction. J Invest Dermatol 121:132–141, 2003***

The S100 proteins comprise a family of EF hand proteins that exist as noncovalently linked homodimers and heterodimers (Donato, 1999). Calcium binding to the EF hands exposes hydrophobic domains, and results in S100 protein activation. The calcium-activated S100 dimer then interacts with specific target proteins. This interaction alters target protein function (Heizmann and Cox, 1998; Donato, 1999). By this mechanism, S100 proteins regulate cell structure, shape, proliferation, and differentiation (Heizmann and Cox, 1998; Donato, 1999). These proteins are thought to be important in epidermal differentiation, as selected members are expressed in epidermis (Hoffmann *et al*, 1994; Tavakkol *et al*, 1994; Boni *et al*, 1997) and the corresponding genes are clustered in the epidermal differentiation complex at chromosomal location 1q21 (Engelkamp *et al*, 1993). Moreover, they are activated by calcium (Donato, 1999), an important regulator of keratinocyte terminal differentiation (Eckert *et al*, 1997). S100A7, also called psoriasin, is expressed at very low levels in normal skin and in cultured keratinocytes; however, expression is markedly increased in hyperproliferative skin diseases, such as psoriasis, suggesting that S100A7 may play a part in disease pathogenesis (Madsen *et al*, 1991, 1992; Hoffmann *et al*, 1994; Algermissen *et al*, 1996). The role of S100A7 in psoriasis is not well understood; however, a putative S100A7 interaction partner, epidermal fatty acid binding protein (EFABP), has been identified in extracts from psoriatic epidermis (Hagens *et al*, 1999a, b). Like S100A7, EFABP is overexpressed in psoriatic epidermis (Siegenthaler *et al*, 1994; Hagens *et al*, 1999a).

In this study we utilize adenoviral expression vectors to express S100A7 and EFABP in cultured normal human keratinocytes as a model to study the localization, distribution, and interaction of these proteins. Our results suggest that S100A7 and EFABP interact, are codistributed within cells, and redistribute to structures at the cell periphery following calcium stimulus. Co-localization with paxillin and α-actinin identify these as adhesion plaque-like peripheral structures. Okadaic acid also promotes this redistribution, suggesting that the process involves a phosphatase-sensitive kinase.

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Abbreviations: Ad, adenovirus; DSP, dithiobis(succinimidylpropionate); DSS, disuccinimidyl suberate; EFABP, epidermal fatty acid binding protein; MOI, multiplicity of infection; PFU, plaque forming units; TE, total extract.

MATERIALS AND METHODS

Cell culture Passage three foreskin keratinocytes were maintained in keratinocyte serum free-medium, supplemented with pituitary extract

and epidermal growth factor. All human tissue samples were collected respecting the Helsinki Principles.

Adenovirus construction For S100A7 adenovirus construction, primers (5′-GCGAATTCATGAGCAACACTCAAGCTGAG (upstream) and 5′-TAGGATCCTGGGTCTC TGGAGGCCCATTG (downstream) were used in a polymerase chain reaction using pET28-S100A7 plasmid (Ruse *et al*, 2001) as template. The underlined sequences in the primers designate a *Eco*RI and a *Bam*HI restriction sites. The resulting polymerase chain reaction product was cloned into pZERO, sequenced, and the S100A7 insert was excised using *Eco*RI and *Bam*HI, and transferred to *Eco*RI/*Bam*HI-restricted pCA3 (Microbix Biosystems, Toronto, Canada). pCA3 contains a CMV promoter and an SV40 polyadenylation signal. The resulting plasmid, pCA3-S100A7, was cotransfected with pJM17 adenovirus backbone vector (Microbix Biosystems) into 293 cells. After recombination, selected plaques were isolated, purified, and amplified in 293 cells to yield Ad5-S100A7. For EFABP adenovirus construction, human RNA was isolated from human keratinocytes, reverse transcribed using a first strand cDNA synthesis kit (Roche Biochemicals, Indianapolis, IN, USA), and the resulting single-stranded cDNA was amplified in a polymerase chain reaction using EFABP specific primers (5′-TTGAATTCATGGCCACAGTTCAGCAGC, upstream; 5′-CGTCTAGATTATTCTACTTTTTCATAGATCC, downstream). The *Eco*RI and *Xba*I restriction sites are underlined. The polymerase chain reaction product was subcloned to pZERO and sequenced. The EFABP cDNA was excised using *Eco*RI/*Xba*I and transferred to *Eco*RI/*Xba*I-restricted pCA3 to create pCA3-EFABP. Co-transfection of 293 cells with pCA3-EFABP and pJM17 yielded recombinant virus, Ad5-EFABP.

To add the HA epitope at the amino-terminus of S100A7 or EFABP, HA-encoding complementary oligonucleotides (5′-TCGACATGTA-CCCATACGACGTCCCAGACTACGCTG and 5′-AATTCAGCGTAGT-CTGGGACGTCGTATGGGTACATG) were synthesized. The underlined sequences contain partial *Sal*I and *Eco*RI sites that were used to insert the HA sequence at the amino terminus of S100A7 and EFABP to create pCA3-HA-S100A7 and pCA3-HA-EFABP. Using an identical approach, FLAG-S100A7 and FLAG-EFABP viruses were produced using FLAG-encoding complimentary oligonucleotides 5′-TCGACATGGACTACA-AGGACGACGACGACAAGG and 5′-AATTCCTTGTCGTCGTCGTC CTTGTAGTCCATG. The underlined sequences identify partial *Sal*I and *Eco*RI restriction sites that were used to insert the FLAG sequence. The resulting adenoviruses include Ad5-S100A7, Ad5-EFABP, Ad5-HA-S100A7, Ad5-HA-EFABP, Ad5-FLAG-S100A7, and Ad5-FLAG-EFABP. Keratinocytes were treated at adenovirus infection levels (multiplicity of infection, MOI = viral particles per cell) designed to infect a high percentage of cells (>90%) without causing virus-dependent toxicity. The percent of cells infected was evaluated using a vector, Ad5-GFP, which encodes green fluorescent protein (Dashti *et al*, 2001).

Immunoprecipitation Near-confluent keratinocyte cultures, grown in ten 50 cm^2 dishes in keratinocyte serum free medium, were infected with the appropriate S100A7- or EFABP-encoding adenoviruses in the presence of 2.5 μg polybrene per mL. After 24 h, the medium was replaced with 10 mL of fresh keratinocyte serum free medium, and the cells were incubated for 24 h. The cells were then washed with phosphate-buffered saline (PBS) and treated for 60 min with 1 mM of the bifunctional cross-linkers, dithiobis(succinimidylpropionate) (DSP) or disuccinimidyl suberate (DSS), obtained from Pierce (Pierce, Rockford, IL, USA), and then washed and scraped into 1 mL of PBS and centrifuged. The pellet was resuspended in 500 μL of lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediamine tetraacetic acid, 1 mM ethyleneglycol-bis-(β-aminoethylether)-*N,N,N′,N′*-tetraacetic acid, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerolphosphate, 1 mM Na_3VO_4, 1 μg per mL leupeptin, 1 mM phenylmethylsulfonyl fluoride) and sonicated. Disrupted cells were centrifuged at 14,000 × *g* at 4°C, and the supernatant was saved as whole cell lysate. Protein concentrations were determined by Bradford assay.

One milligram of total protein was incubated with rabbit anti-S100A7 antibody, rat anti-HA high affinity antibody (Roche Biochemicals, 1.5 μg), or mouse anti-FLAG (Sigma, 5 μg) (Sigma-Aldrich St. Louis, MO, USA) for 3 h at 4°C. Protein–antibody complexes were then precipitated with protein (A + G)-agarose (SantaCruz Biotechnology, Santa Cruz, CA, USA) for 1 h at 4°C. Precipitates were washed three times with lysis buffer, resuspended in 50 μL Laemmli sample buffer and boiled for 5 min. The precipitated samples were electrophoresed, in parallel, with 20 μg of whole cell lysate on a 15% acrylamide gel, and the proteins were transferred to PVDF membrane. For S100A7 detection, the blots were incubated with polyclonal rabbit anti-S100A7 antibody (1:1000) overnight, washed, and then incubated with horseradish peroxidase-conjugated donkey anti-rabbit IgG (Amersham, 1:10,000) (Amersham, Piscataway, NJ, USA) for 1 h. For HA-EFABP detection, the blots were incubated with horseradish peroxidase-conjugated rat anti-HA (Roche, 1:400) for 1 h. For detection of S100A9, the blots were incubated with goat anti-S100A9 (Santa Cruz, 1:500) followed by horseradish peroxidase-conjugated donkey anti-goat IgG (Santa Cruz, 1:10,000). All blots were washed thoroughly after incubation with the secondary antibody and exposed to ECL detection reagents (Amersham).

Immunofluorescence Keratinocytes were plated on glass coverslips, and infected with either Ad5-HA-S100A7 (15 MOI) or Ad5-FLAG-EFABP (10 MOI), or coinfected with both adenoviruses. At 48 h postinfection, the cells were treated with 0.3 mM calcium chloride and 10 μM A23187 ionophore (Sigma) for up to 90 min. Following treatment, the cells were fixed for 1 h in 2% paraformaldehyde and permeabilized for 30 min in 100% ice-cold methanol. The following primary and secondary antibodies were used for detection: fluorescein isothiocyanate-conjugated rat anti-HA (Roche, 1:50), Cy3-conjugated mouse anti-FLAG (Sigma, 1:100), rabbit anti-HA (Sigma-Aldrich, 1:100), mouse anti-α-actinin (Sigma-Aldrich, 1:200), Cy3-conjugated sheep anti-mouse IgG (Sigma, 1:500), and AlexaFluor 488-conjugated goat anti-rabbit IgG (Molecular Probes, 1:1000) (Molecular Probes, Eugene, OR, USA).

Confocal microscopy Keratinocytes were plated on 22 × 22 mm glass coverslips (Fisher Scientific, Pittsburgh, PA, USA). The cells were infected with Ad-HA-S100A7 (15 MOI) and 48 h postinfection the cells were treated with 0.3 mM Ca^{2+} and 10 μM A23187 ionophore for 60 min. Following treatment, the cells were fixed for 1 h in 2% paraformaldehyde and permeabilized for 30 min in 100% ice cold methanol. The primary antibodies used to costain the cells were rabbit anti-HA (Sigma; 1:100) and either of the following antibodies from BD Transduction Laboratories (Transduction Laboratories, San Diego, CA, USA) for different organelle markers: mouse anti-BiP (1:50), mouse anti-LAMP1 (1:50), or mouse anti-paxillin (1:500). Then the cells were incubated with the secondary antibodies AlexaFluor488-conjugated goat anti-rabbit (Molecular Probes; 1:1000) and Cy3-conjugated sheep anti-mouse (Sigma; 1:500). Images were analyzed using a confocal microscope equipped with an argon laser source (model LSM510; Carl Zeiss).

Immunohistochemistry Uninvolved and involved psoriatic epidermis was fixed overnight at 4°C in 2% paraformaldehyde and sectioned. Nonspecific antibody binding was blocked by incubating sections for 30 min at 25°C with 1.5% serum (rabbit, S-5000; Vector Laboratories, Burlingame, California). Blocked sections were then incubated with preimmune serum or S100A7 isoform-specific antibody (diluted 1:100 in PBS) for 1 h at 25°C, rinsed with PBS, and incubated with biotin-labeled secondary antibody (goat anti-rabbit IgG, Vector Laboratories) for 1 h at 25°C in PBS. After washing, the sections were incubated for 30 min with avidin–biotin peroxidase complex. The sections were rinsed, stained with 3,3′-diaminobenzidine (SK-4100, Vectastain Kit) (Vector Laboratories, Burlingame, CA, USA) and counterstained with hematoxylin.

RESULTS

S100A7/EFABP localization S100A7 and EFABP are highly overexpressed in psoriatic tissue, but are not expressed in undifferentiated cultured human keratinocytes. Thus, cultured cells provide a useful null background in which to deliver S100A7 and/or EFABP and to study their function. To express these proteins, cells were infected with S100A7- or HA-EFABP-encoding adenoviruses, and appearance of the corresponding protein was monitored by immunoblot. As shown in **Fig 1**(***A***), steady-state expression of S100A7 and HA-EFABP was observed at 48 h. We next monitored the subcellular distribution of FLAG-EFABP and HA-S100A7 using confocal microscopy. In cells maintained in low calcium-containing medium, HA-S100A7 (green) and FLAG-EFABP (red) were distributed in the cytoplasm with decreased staining towards the cell periphery (**Fig 1*B***, left panel). The combined image confirms this colocalization (yellow). The enlarged orthogonal combined image confirms colocalization in the cytoplasm of the cell (white arrow), and also identifies a pool of S100A7 that is

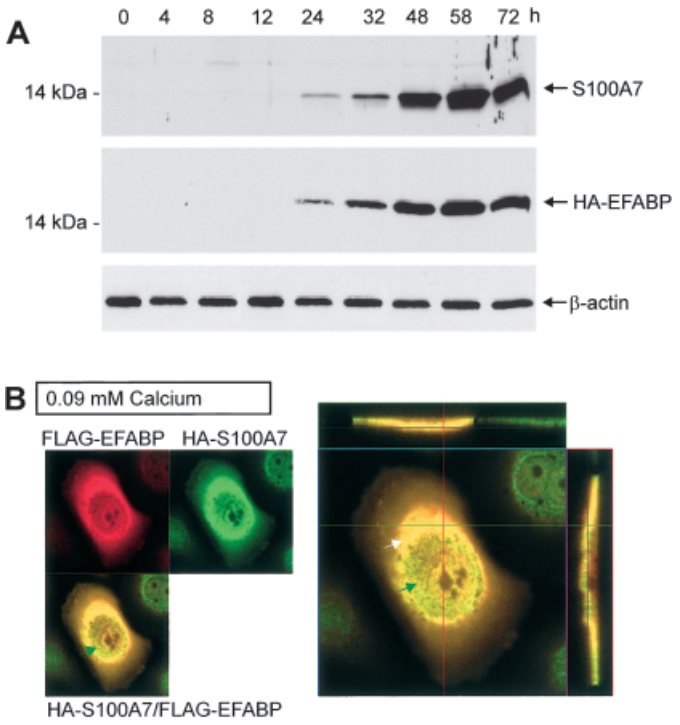

Figure 1. Time course of HA-EFABP and S100A7 expression in keratinocytes. (*A*) Near-confluent (70%) cultures were infected with 15 MOI of Ad5-S1007 or 10 MOI of Ad5-HA-EFABP at time 0 in medium containing 0.09 mM calcium. Whole cell extracts were prepared at the indicated time points postinfection and S100A7 and HA-EFABP were detected by immunoblot. β-actin serves as a control to normalize protein loading and was detected using mouse anti-β-actin (Sigma, 1:10,000) followed by horseradish peroxidase-conjugated sheep anti-mouse IgG (Amersham, 1:10,000). (*B*) Keratinocytes were infected as in (A). After 48 h, the cells were fixed, permeabilized, and HA-S100A7 and FLAG-EFABP were detected by immunofluorescence and visualized by confocal microscopy. The left panels show single plane images (green, HA-S100A7; red, FLAG-EFABP; yellow, combined), whereas the enlarged panel on the right shows the combined orthogonal image.

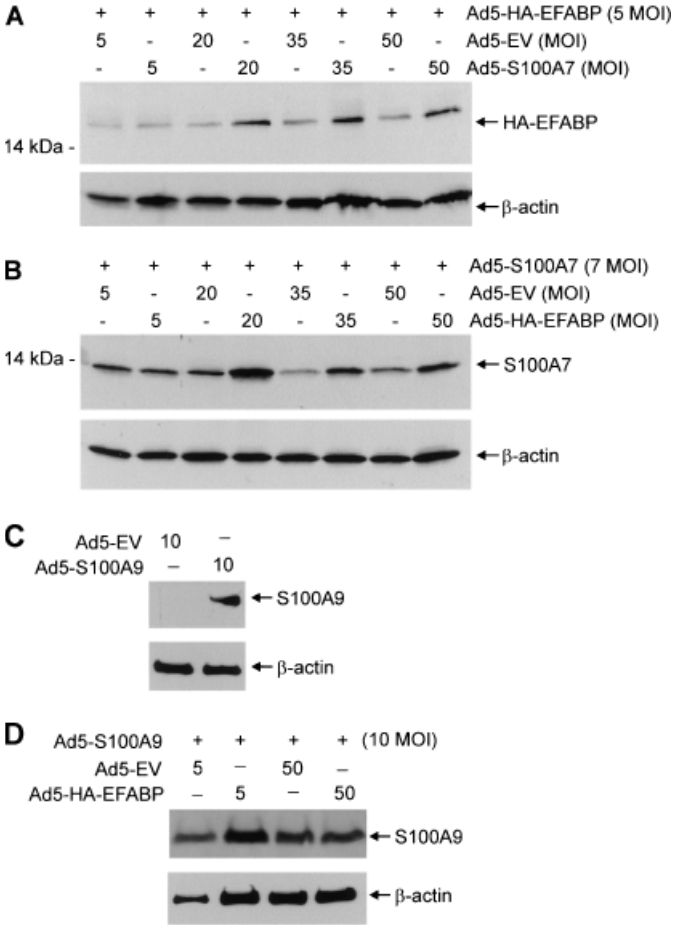

Figure 2. Mutual stabilization of S100A7 and EFABP. (*A*) Stabilization of HA-EFABP by S100A7. Cells were infected with 5 MOI of Ad5-HA-EFABP at time 0, followed at 24 h by infection with the indicated MOI of Ad5-S100A7. After an additional 24 h, extracts were prepared and assayed for HA-EFABP level by immunoblot. (*B*) Stabilization of S100A7 by HA-EFABP. Cells were infected with 7 MOI of Ad5-S100A7 at time 0, followed at 24 h by infection with the indicated MOI of Ad5-HA-EFABP. After an additional 24 h, extracts were prepared and S100A7 level was assayed by immunoblot. (*C*) Expression of S100A9 in keratinocytes. Fifty percent confluent keratinocyte cultures were infected at time 0 with 10 MOI of either Ad5-EV or Ad5-S100A9, and at 48 h postinfection whole cells extracts were assayed for S100A9 by immunoblot. (*D*) EFABP does not stabilize S100A9. Cells were infected with 10 MOI of Ad5-S100A9 at time 0, followed at 24 h by infection with the indicated MOI of HA-EFABP. After an additional 24 h, extracts were assayed for S100A9 level by immunoblot. β-actin levels are shown as a control to normalize loading.

localized in perinuclear structures in the absence of EFABP (green arrow).

S100A7 and EFABP interaction Based on previous reports by Siegenthaler and coworkers (Hagens *et al*, 1999a, b), which suggested that S100A7 and EFABP interact in psoriatic epidermal extracts, we examined the interaction of S100A7 and EFABP in cultured cells. Keratinocytes were infected with HA-EFABP expressing adenovirus and incubated for 24 h. Cells were then secondarily infected with increasing levels (MOI) of empty adenovirus or S100A7 expressing adenovirus. The cells were harvested at 48 h and EFABP level was measured. **Figure 2**(***A***) shows that S100A7 increases EFABP level across a range of S100A7 concentrations. We confirmed this result in an inverse experiment in which the cells initially expressed S100A7, and were subsequently infected with empty virus or HA-EFABP encoding virus (**Fig 2*B***). These results suggest that S100A7 and HA-EFABP stabilize one another. To determine whether this effect is specific for S100A7, we measured the ability of HA-EFABP to stabilize another S100 protein, S100A9. We first confirmed that Ad5-S100A9 produces S100A9 in keratinocytes. Cells were infected with Ad5-S100A9 or empty vector (Ad5-EV). After 48 h, cells were harvested and assayed for S100A9 protein. **Figure 2**(***C***) shows that S100A9 is expressed. To examine HA-EFABP effects on S100A9 level, cells were infected with S100A9 encoding adenovirus, followed 24 h later by infection with empty virus or HA-EFABP producing virus. After an additional 24 h, the cells were harvested and S100A9 level was assessed by immunoblot. **Figure 2**(***D***) shows that, when normalized to β-actin, S100A9 levels are not increased (stabilized) by HA-EFABP.

To study further S100A7/EFABP interaction, cells were infected with Ad5-S100A7 and Ad5-HA-EFABP, and after 48 h extracts were prepared for immunoprecipitation; however, efforts to coprecipitate S100A7 and EFABP from these extracts were not successful (not shown). We next repeated the infection and at 48 h postinfection, treated cells for 60 min with 1 mM DSS, a noncleavable bifunctional cross-linking agent. This agent is widely used to cross-link closely juxtaposed proteins in cells. Total extract (TE) and anti-HA immunoprecipitate (IP: α-HA) were prepared and electrophoresed for immunoblot. As a control to assure that HA-EFABP was precipitated, the blot

shown in **Fig 3**(***A***) was incubated with anti-HA. HA-EFABP monomer, as well as higher molecular weight cross-linked species (asterisk), was detected in each sample, indicating that anti-HA-EFABP precipitation was successful. To assess whether S100A7 coprecipitates with HA-EFABP, a parallel blot was incubated with anti-S100A7. As shown in **Fig 3**(***B***), S100A7 monomer and higher molecular weight immunoreactive species (asterisk) were present in the TE. The anti-HA-EFABP-precipitated sample (IP: α-HA) was enriched in high molecular weight S100A7-immunoreactive material (**Fig 3*B***, right panel), indicating that S100A7 is associated with HA-EFABP. As expected, S100A7 monomer was not precipitated. The double asterisks indicate nonspecifically precipitated bands. Neither HA-EFAPB nor S100A7 is precipitated from cells infected with empty virus (**Fig 3*A,B***).

These findings were confirmed by the inverse experiment showing that S100A7 precipitates EFABP. Cells were infected with Ad5-FLAG-S100A7 and Ad5-HA-EFABP for 48 h, and treated with or without DSS cross-linker for 60 min. TE and anti-FLAG-S100A7 immunoprecipitate (IP: α-FLAG) was electrophoresed and prepared for immunoblot. The blot was then incubated with anti-HA-EFABP. HA-EFABP was detected in the TE, both as a monomer and as higher molecular weight forms (**Fig 3*C***, TE). HA-EFABP monomer would not be expected to precipitate with FLAG-S100A7. No precipitation was observed in cells infected with control empty virus.

We wanted to determine whether S100A7 and EFABP monomer could be released from the high molecular weight material shown in **Fig 3**(***A–C***). We expressed S100A7 and HA-EFABP in keratinocytes. After 48 h, the cells were treated for 60 min with 1 mM DSP, a cleavable cross-linking agent. Extracts were precipitated with anti-HA-EFABP, boiled with reducing agent to cleave the cross-link, electrophoresed, and incubated with antibodies specific for HA-EFABP or S100A7 (**Fig 4*A***). The upper blot, incubated with anti-HA-EFABP, shows that anti-HA precipitates HA-EFABP. The lower blot shows that S100A7 can be released from the precipitated complex, indicating that it coprecipitated with HA-EFABP. Precipitation with nonspecific antibody did not precipitate either protein (not shown). The converse experiment is shown in **Fig 4**(***B***), and confirms that HA-EFABP coprecipitates with S100A7.

Calcium treatment promotes S100A7 and EFABP redistribution to peripheral structures As shown in **Fig 1**(***B***), in cells maintained in low calcium medium, S100A7 and HA-EFABP colocalize diffusely throughout the cell cytoplasm. As S100 proteins are activated by calcium (Heizmann and Cox, 1998), and calcium is a potent keratinocyte differentiation stimulus, we examined the effects of calcium on S100 protein subcellular localization. We used a classical method of increasing intracellular calcium in keratinocytes in which cells are incubated with calcium in the presence of ionophore (Green, 1980). **Figure 5**(***A***) (0 min) shows that in cells grown in 0.09 mM calcium-containing medium, S100A7 and EFABP colocalize in the cytoplasm. Shifting the cells to medium containing 0.3 mM calcium and 10 μM A23187 results the formation of peripheral structures that appear as dark spots in bright field images (**Fig 5*A***, arrows). Both S100A7 and EFABP are enriched in these structures (arrows). At lower magnification (**Fig 5*B***), it is possible to determine that these structures form in 28% of cells at 60 min after calcium/A23187 treatment (arrows). **Figure 5**(***C***) shows that S100A7 and EFABP also distribute to these structures when expressed alone, indicating that this distribution does not require that both proteins be present. Based on the cell culture results presented above, we hypothesized that S100A7 might distribute to the plasma membrane *in vivo*. Indeed, staining of normal epidermis with anti-S100A7 shows a diffuse distribution in basal cells with progressively more peripheral S100A7 localization in the high suprabasal layers. A similar pattern is observed in psoriatic epidermis, except that the peripheral localization is more dramatic (**Fig 6**).

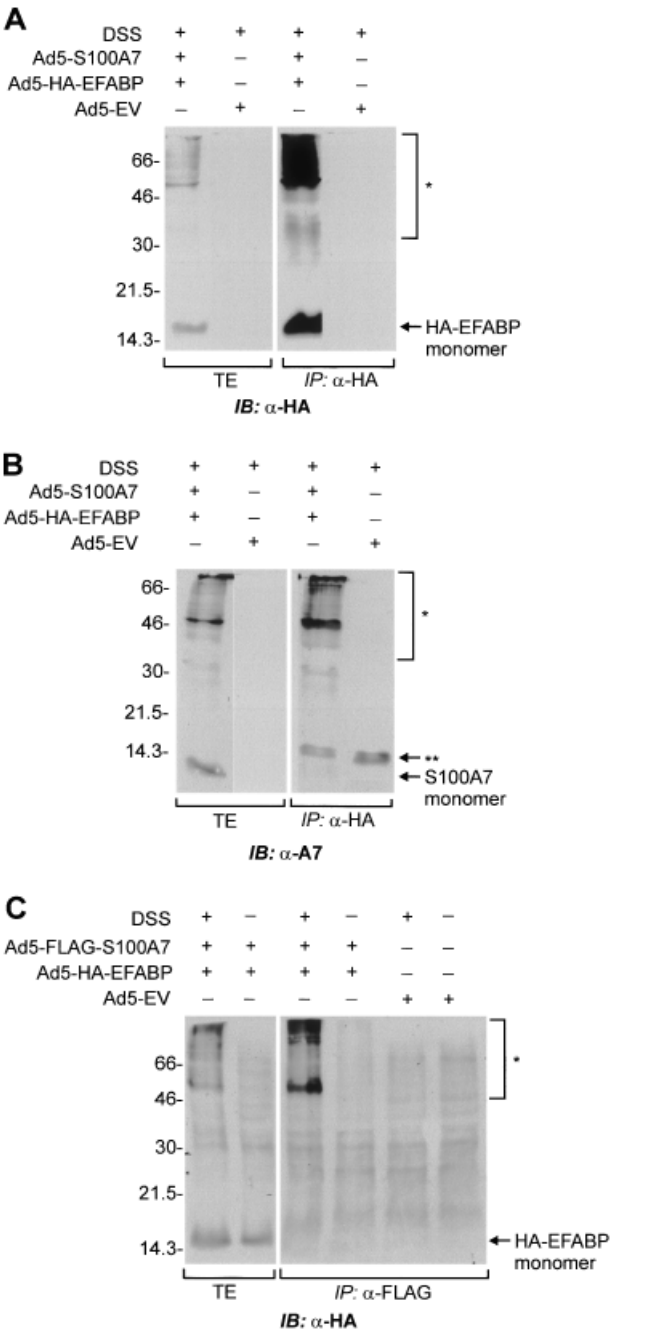

Figure 3. Co-precipitation of S100A7 and HA-EFABP. (*A*) Keratinocytes were infected with 25 MOI of Ad5-EV, 15 MOI Ad5-S100A7, or 10 MOI of Ad5-HA-EFABP for 48 h. Cells were subsequently treated with DSS cross-linker. TE or anti-HA immunoprecipitate were electrophoresed and HA-EFABP was detected by immunoblot. High molecular weight HA-EFABP containing complexes are indicated by the asterisk. (*B*) An identical parallel blot to that shown in the above panel was incubated with anti-S100A7. High molecular weight complexes containing S100A7 are indicated by the asterisk. Double asterisks indicate nonspecific bands. (*C*) Keratinocytes were infected with 25 MOI of Ad5-EV, 15 MOI of Ad5-FLAG-S100A7, or 10 MOI of Ad5-HA-EFABP for 48 h. TE and anti-FLAG immunoprecipitate were electrophoresed and HA-EFABP was detected by immunoblot. HA-EFABP migrates as a higher molecular weight complex marked by the asterisk. Details are provided in *Materials and Methods*.

S100A7 redistribution in cultured keratinocytes We used confocal microscopy to examine the redistribution in cultured

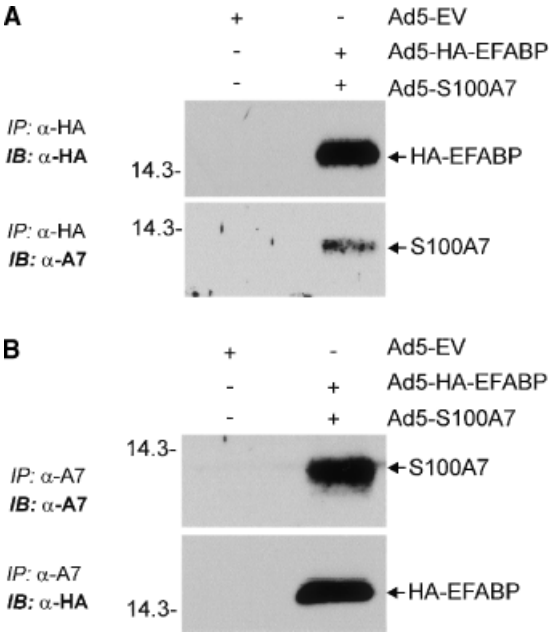

Figure 4. Release of coprecipitated S100A7 and HA-EFABP monomers. Keratinocytes were infected with 15 MOI of Ad5-S100A7, 25 MOI of Ad5-EV, or 10 MOI of Ad5-HA-EFABP as indicated for 48 h. The cells were then incubated for 1 h with 1 mM of cleavable DSP cross-linker and extracts were prepared in reducing agent-containing gel buffer to cleave the DSP-dependent cross-link. **A** Immunoprecipitation with anti-HA, followed by electrophoresis and blotting with anti-HA or anti-A7. (*B*) Immunoprecipitation with anti-A7 followed by electrophoresis and blotting with anti-A7 or anti-HA. Co-immunoprecipitation was not observed when protein cross-linker treatment was omitted (not shown).

cells in greater detail. As shown in **Fig 1**(***B***), in cells maintained in low calcium conditions, S100A7 and HA-EFABP colocalize in the perinuclear cytosol. In addition, perinuclear structures stain exclusively for S100A7 (see **Fig 1*B***). Treatment with 0.3 mM calcium + 10 μM A23187 results in a substantial redistribution of both HA-S100A7 (green) and FLAG-EFABP (red) to peripheral structures (**Fig 7**). The combined image (yellow) shows substantial colocalization of S100A7 and EFABP in these structures. An enlarged orthogonal image is shown on the right. The domains are actually protrusions extending downward from the cell surface. The yellow color indicates the presence of both HA-S100A7 and FLAG-EFABP. In addition, EFABP-negative/S1007-positive perinuclear vesicular staining is also observed in 0.3 mM calcium/A23187-treated cells (green arrows).

S100A7 colocalizes with α-actinin To learn more about the cell periphery structures, cells were infected with Ad5-S100A7, treated for 60 min with calcium, and then costained with anti-HA-S100A7 and anti-α-actinin (**Fig 8**). The α-actinin (red) and HA-S100A7 (green) images indicate that both proteins are present in these structures and the combined image (yellow) indicates that they colocalize. The orthogonal combined confocal image (right panel) shows that S100A7 and α-actinin colocalize in the peripheral structures (white arrows). Additional studies confirm that EFABP also colocalizes at these sites (not shown). This image also shows that α-actinin is not present with S100A7 in the perinuclear vesicles (green arrows).

Localization with other markers To assign further the location of S100A7, cells maintained in 0.09 mM calcium or 0.3 mM calcium/A23187, were costained for S100A7 and markers associated with various subcellular organelles (**Fig 9**). BiP/GRP78 is a marker of endoplasmic reticulum (Rao *et al*, 2002). Our analysis shows that in control cells, some of the S100A7 localizes with BiP/GRP78 in perinuclear tubular endoplasmic reticulum-like structures. Upon treatment with calcium and ionophore, the endoplasmic reticulum fragments into vesicles. Some of these vesicles stain with BiP, some stain with S100A7, and some stain for both (see insert). In addition, a strong colocalization of S100A7 and paxillin, a focal adhesion organizing protein (Lin *et al*, 1995), is observed in calcium/A23187-treated cells. This finding is consistent with the colocalization of S100A7 with α-actinin shown in **Fig 8**, as paxillin and α-actinin are known to participate in complex formation in focal adhesions. In contrast, S100A7 does not localize with LAMP-1, a late endosomal/lysosomal marker (Karlsson and Carlsson, 1998), suggesting that it is not being targeted for degradation.

Kinase and phosphatase regulators influence S100A7/EFABP redistribution The above results show that calcium promotes redistribution of S100A7 and EFABP. To examine further the regulation of redistribution, we expressed HA-S100A7 and FLAG-EFABP, and then treated with 12-*O*-tetradecanoyl-phorbol-13-acetate or okadaic acid for 60 min. 12-*O*-tetradecanoyl-phorbol-13-acetate did not cause redistribution of S100A7 or EFABP—both remained colocalized in a perinuclear region (nucleus is circled) (**Fig 10**). In contrast, treatment with okadaic acid caused rapid redistribution of both proteins to cell periphery structures (arrows).

DISCUSSION

S100 proteins in epidermis S100 proteins comprise a 16 member multigene family that encode calcium-activated signaling proteins (Donato, 2001). S100 proteins exist as dimers in cells (Donato, 2001), and each monomer contains two calcium-binding EF-hand motifs (Schafer and Heizmann, 1996). These motifs are separated by a flexible hinge region, and flanked by hydrophobic regions (Schafer and Heizmann, 1996). The C-terminal EF-hand is a canonical calcium-binding loop containing 12 amino acids, whereas the N-terminal motif is a noncanonical calcium-binding motif that includes 14 amino acids and has a lower affinity for calcium (Krebs *et al*, 1995). It has been proposed that increased intracellular calcium causes a change in S100 dimer conformation and permits the dimer to interact with target protein(s). This interaction then alters target protein function to produce a biologic response. Thus, two important goals are identification of S100 target proteins and sites of intracellular action (Heizmann and Cox, 1998). At least six members of this multiprotein family are expressed in epidermis, including S100A2, S100A7, S100A8, S100A9, S100A10, and S100A11 (Boni *et al*, 1997; Robinson *et al*, 1997; Donato, 2001). Expression of selected S100 proteins is increased in epidermal disease. For example, S100A7, S100A8, and S100A9 are overexpressed in psoriasis (Madsen *et al*, 1991; Saintigny *et al*, 1992). S100A7 expression is also markedly increased in other hyperproliferative skin diseases, including atopic dermatitis, mycosis fungoides, and Darier's disease (Madsen *et al*, 1991; Algermissen *et al*, 1996). These findings indicate that S100A7 is a marker of these diseases, and also suggest a potentially important, but unproven, role for S100A7 in the genesis and/or maintenance of skin disease.

Evidence for intracellular interaction of S100A7 and EFABP Previous studies using extracts prepared from psoriatic-involved epidermis provided an important clue that epidermal fatty acid binding protein might be an S100A7 target (Hagens *et al*, 1999a, b). Interestingly, like S100A7, EFABP is expressed at low levels in normal skin but is overexpressed in psoriatic epidermis (Madsen *et al*, 1992; Siegenthaler *et al*, 1993, 1994). To study this interaction in cultured keratinocytes, we

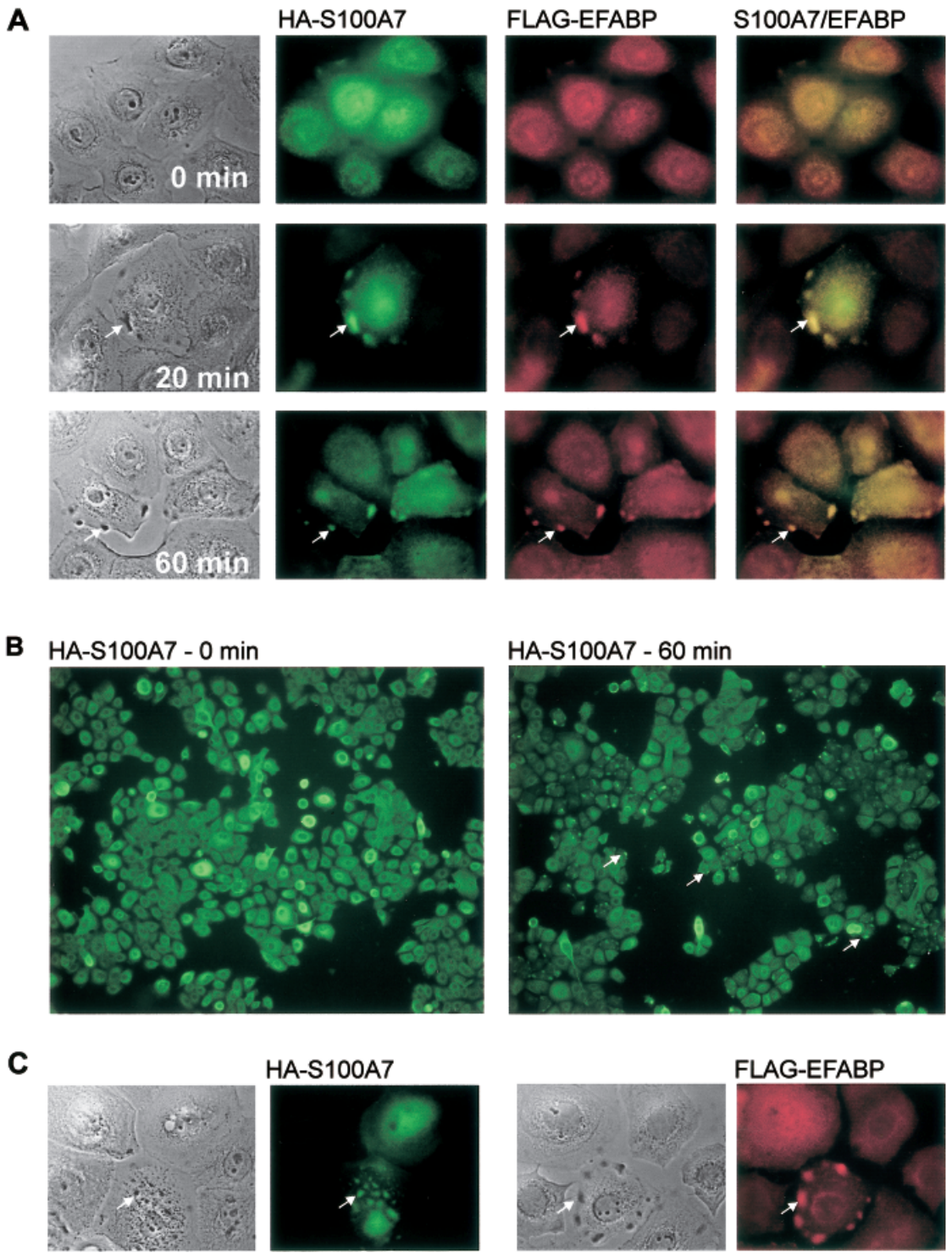

Figure 5. HA-S100A7 and FLAG-EFABP distribution in calcium-treated cells. (*A*) Keratinocytes were infected with 15 MOI Ad5-HA-S100A7 and 10 MOI of Ad5-FLAG-EFABP. At 48 h postinfection, keratinocytes were treated with 0.3 mM calcium and 10 μM A23187 for 0, 20, and 60 min. HA-S100A7 (green) and FLAG-EFABP (red) were detected by fluorescence microscopy. The S100A7/EFABP column shows the combined green and red images—yellow indicates colocalization. The *arrows* indicate localization of S100A7 and EFABP in peripheral structures. (*B*) Cells were infected with 15 MOI Ad5-HA-S100A7. After 48 h, the cells were treated for 0 or 60 min in 0.3 mM calcium/10 μM A23187-containing medium, fixed and stained with anti-S100A7. S100A7 redistributed (e.g., *arrows*) in 28% of the cells at 60 min of treatment (calculated as an average five representative fields, ×40 objective). (*C*) Cells were infected with HA-S100A7 encoding adenovirus (left panels) or FLAG-EFABP encoding adenovirus (right panels). After a 60 min treatment with 0.3 mM calcium/A23187 cells were fixed and stained, respectively, with anti-HA (green) or anti-FLAG (red).

expressed S100A7 and EFABP in cultured human keratinocytes. Initial microscopic studies showed that the expressed S100A7 and EFABP proteins colocalize in cells. Subsequent biochemical studies show that increased S100A7 expression results in increased levels of EFABP and vice versa—suggesting a stabilizing interaction. This effect was specific for S100A7, as a similar increase in S100A9, another S100 protein overexpressed in psoriatic epidermis (Siegenthaler *et al*, 1997), was not observed in the presence of EFABP. Characterization of FLAG-S100A7-associated complexes in human cultured keratinocytes using a

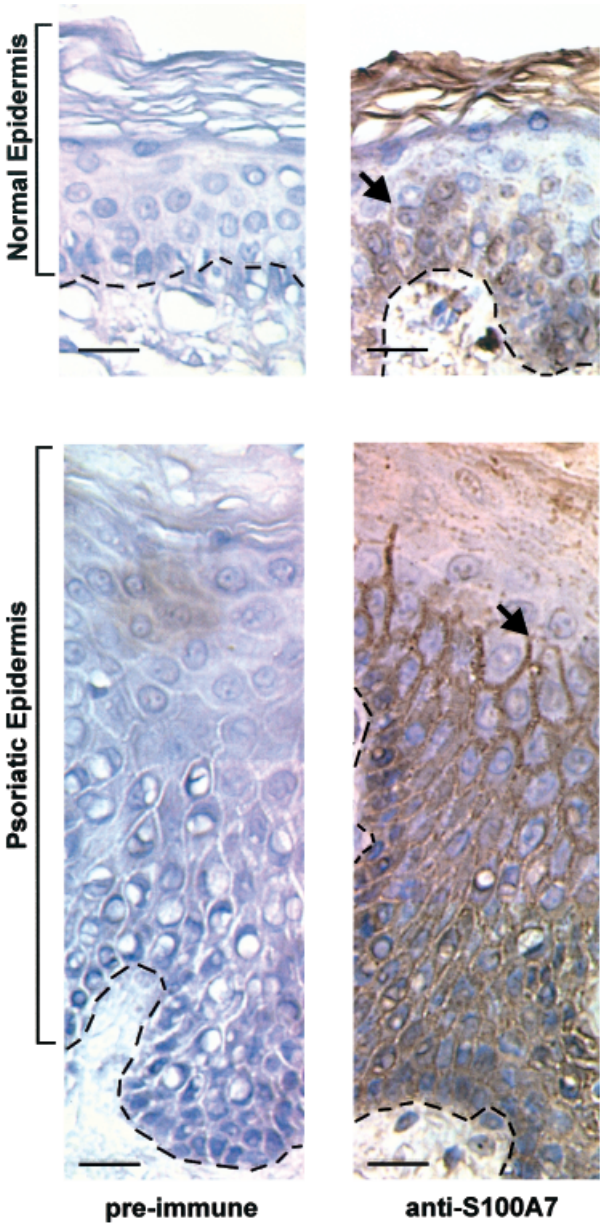

Figure 6. Distribution of S100A7 in normal and psoriatic epidermis. Sections from uninvolved (normal epidermis—*top panels*) and involved (psoriatic epidermis—*bottom panels*) were incubated with preimmune or anti-S100A7 (see *Materials and Methods*). The *arrows* indicate areas of S100A7 at the cell periphery. The dashed line traces the basal lamina. *Scale bars* = 20 μm. The staining of the cornified layer is nonspecific.

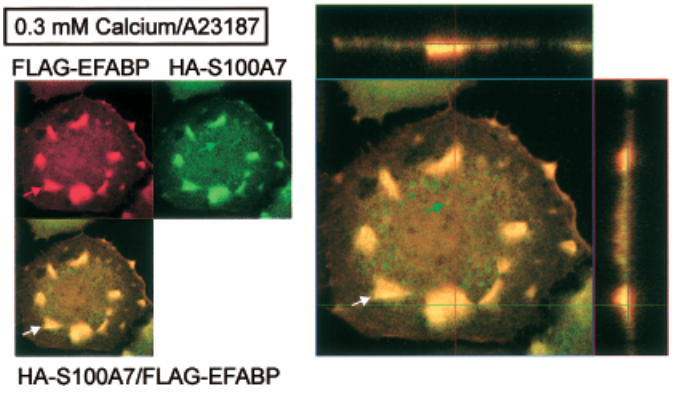

Figure 7. Confocal images—calcium mobilization of HA-S100A7 and FLAG-EFABP. Keratinocytes were infected with 15 MOI Ad5-HA-S100A7 and 10 MOI of Ad5-FLAG-EFABP and maintained in low calcium (0.09 mM) containing medium. At 48 h postinfection, keratinocytes were treated with 0.3 mM calcium and 10 μM A23187 for 60 min. Following fixation and permeabilization, HA-S100A7 (green) and FLAG-EFABP (red) were detected by immunofluorescence and the images were visualized using confocal imaging. The *white arrows* indicate HA-S100A7 and FLAG-EFABP colocalization in plasma membrane structures that make contact with the underlying substratum following calcium/A23187 treatment. The *green arrows* identify S100A7-enriched intracellular vesicles, and the *red arrow* indicates EFABP peripheral structures, *white arrows* indicate S100A7/EFABP colocalization.

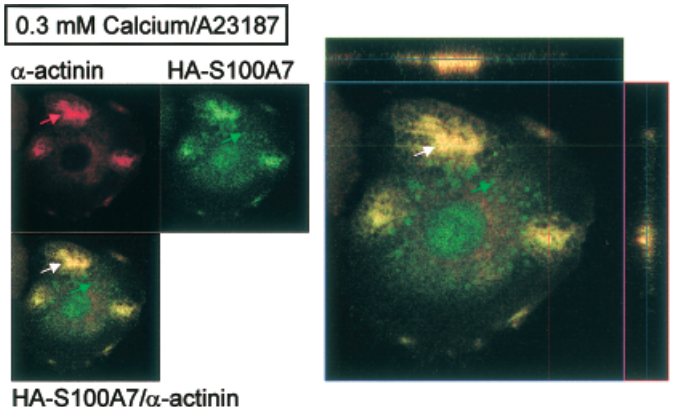

Figure 8. Confocal images—colocalization of HA-S100A7 and α-actinin in calcium-treated cells. Keratinocytes were infected with 15 MOI Ad5-HA-S100A7 and at 48 h postinfection were treated with 0.3 mM calcium/10 μM A23187 for 60 min. Following fixation and permeabilization, HA-S100A7 (green) was detected by immunofluorescence. In addition, the cells were costained to detect α-actinin (red). The picture composite shows individual α-actinin and HA-S100A7 distribution, and the combined (α-actinin/HA-S100A7) image in cells maintained in high calcium. The accompanying orthogonal projection of the combined image is shown at the right. The *white arrows* indicate α-actinin and HA-S100A7 colocalization in peripheral structures. The *green arrows* indicate S100A7-enriched intracellular vesicles, and the *red arrow* indicates α-actinin in peripheral structures.

cell membrane-permeable noncleavable cross-linker (DSS) followed by immunoprecipitation with anti-FLAG, revealed high molecular weight protein complexes containing HA-EFABP. Likewise, precipitation of HA-EFAPB resulted in S100A7 coprecipitation. These studies suggest a specific S100A7/EFABP interaction. Although we believe that it is a physiologically important interaction, it may be a transient interaction, as we do not observe coprecipitation of S100A7-EFABP complexes in cells not treated with cross-linking agent. These findings contrast slightly with those of Siegenthaler and coworkers (Hagens *et al*, 1999a) who reported that S100A7 and EFABP formed a complex that could be precipitated without addition of cross-linker (Hagens *et al*, 1999a); however, these investigators prepared precipitates directly from psoriatic tissue and not from cultured cells. Thus, it is possible that these proteins are modified *in vivo*, or an additional protein is expressed *in vivo* that is important for stabilizing the complex. In fact, two forms of S100A7 have been reported that may represent conformational variants and it has been speculated that one of these forms may have a reduced ability to interact with EFABP (Burgisser *et al*, 1995). Moreover, it has been reported that Zn^{2+} ion can cause the complex to dissociate (Hagens *et al*, 1999a), thus it is possible that the ion sensitivity may differ in cultured cells; however, on balance, our studies and those of Siegenthaler and

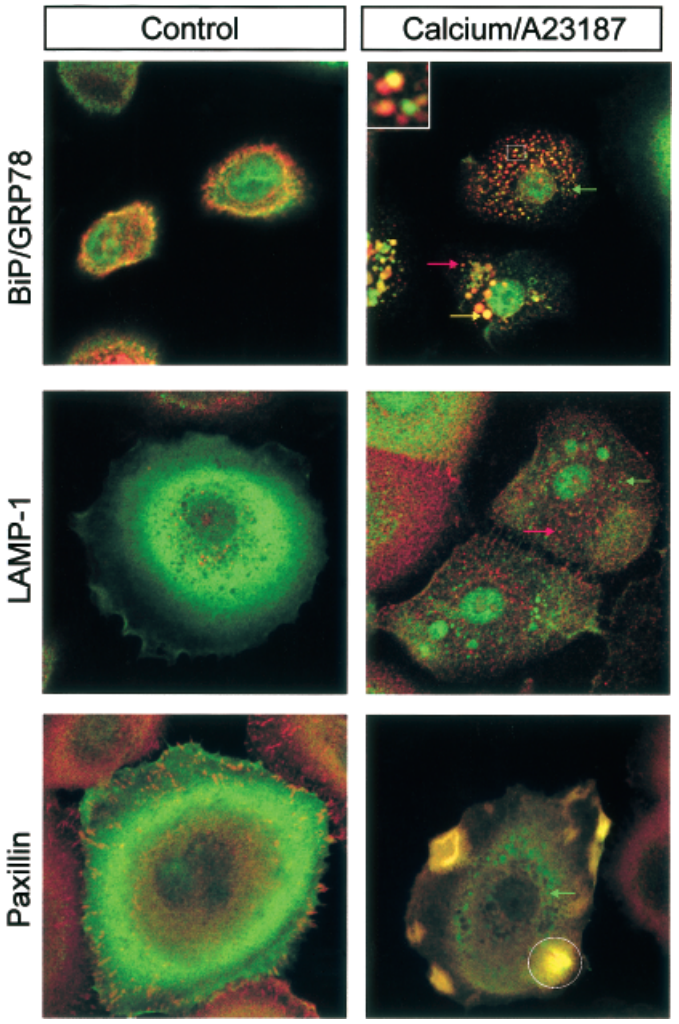

Figure 9. Subcellular localization of HA-S100A7 and markers of intracellular compartments. Keratinocytes were infected with 15 MOI Ad5-HA-S100A7 and at 48 h postinfection were treated with 0.09 mM calcium (Control) or 0.3 mM calcium/10 μM A23187 for 60 min. Following fixation and permeabilization, slides were stained with rabbit anti-HA-S100A7 (green). In addition, the slides were stained with mouse anti-BiP/GRP78, anti-LAMP1 or anti-paxillin (red). *Green arrows* indicate HA-S100A7 immunofluorescence, *red arrows* indicate the presence of the respective marker protein, and *yellow arrows* indicates colocalization of HA-S100A7 with the marker. The images were obtained using confocal microscopy. The circles indicate the location of peripheral structures.

colleagues (Hagens *et al*, 1999a,b) suggest that S100A7 and EFABP are part of a common complex.

S100A7 and EFABP localization and stimulus-dependent redistribution Intracellular localization and distribution are important determinants of protein function. Thus, it is important to identify the intracellular compartment that hosts the presence of S100A7 and EFABP, and whether differentiating agents regulate their distribution. In resting cells, S100A7 and EFABP are codistributed in the cytoplasm. Treatment with calcium, a keratinocyte-differentiating stimulus (Rice and Green, 1979; Hawley-Nelson *et al*, 1980; Tsao *et al*, 1982), results in the redistribution of cytosolic S100A7 and EFABP to defined structures at the cell periphery. These peripheral structures share properties with focal adhesion plaques, including being enriched in paxillin (Petit and Thiery, 2000; Wade *et al*, 2002) and α-actinin (Petit and Thiery, 2000; Edlund *et al*, 2001). The finding that S100A7 is associated with the cell periphery in calcium-treated cultured cells is consistent with a previous demonstration that S100 proteins are covalently cross-linked components of the cornified envelope (Robinson *et al*, 1997), and the observation that S100A7 appears to redistribute from the cytosol to the cell periphery during *in vivo* epidermal differentiation (**Fig 6**). These findings contrast with a previous report indicating that S100A7 remains diffusely distributed in all epidermal layers in psoriasis (Hagens *et al*, 1999a). The explanation for this difference is not clear; however, different antibodies were used for detection in each case.

In addition to the cytoplasmic localization in resting cells, some S100A7 is also present in a perinuclear location in the endoplasmic reticulum. It is not clear why some of the S100A7 is maintained in the endoplasmic reticulum and the remainder is present in the cytoplasm; however, two forms of S100A7 have been reported (Burgisser *et al*, 1995) and these may represent the material we find distributed in the two compartments. Upon calcium treatment the endoplasmic reticulum dissociates into vesicles (Subramanian and Meyer, 1997) and the endoplasmic reticulum-localized S100A7 is retained in these vesicles. Identifying the function of this pool of S100A7 will require additional study.

Functional role of S100A7 Based on these and previously published studies, we can construct a model outlining possible functions of S100A7 in keratinocytes. The present studies clearly show that S100A7 (and EFABP) move to the cell periphery after stimulation of keratinocytes with prodifferentiation agents. Thus, we hypothesize that the vicinity of the plasma membrane is an ultimate site of S100A7 action. This idea is supported by several observations. First, S100A7 is a transglutaminase substrate (Ruse *et al*, 2001) suggesting that it may be a component of the keratinocyte cornified envelope—as are other S100 proteins (Robinson *et al*, 1997; Robinson and Eckert, 1998). To function as such it would need to come in contact with the plasma membrane. Second, S100A7 is known to be secreted and to function as a chemokine (Madsen *et al*, 1991; Jinquan *et al*, 1996; Watson *et al*, 1998), thus, redistribution to the plasma membrane may be a necessary step for export. Third, S100A7 decorates the cell surface in differentiated normal keratinocytes and psoriatic tissue (**Fig 6**). Fourth, S100A7 is localized with proteins that comprise the focal adhesions, including paxillin and α-actinin (**Figs 8 and 9**). These results suggest that S100A7 may function as a shuttle protein to move other proteins, including EFABP, to the cell periphery. Moreover, the association with focal adhesion markers suggests that it may play a part in regulating adhesion, and perhaps motility, during disease, metastasis, and wound healing (Ben Ze'ev *et al*, 1994; Newell *et al*, 1999; Kim *et al*, 2001). The role of EFABP as an S100A7 target, and its role in focal adhesion-like structures are not known; however, EFABP does bind to oleic acid (Hagens *et al*, 1999b). Thus, S100A7 and EFABP could function to transport and/or metabolize fatty acids, including oleic acid (Hagens *et al*, 1999b). Other S100 proteins have also been implicated in fatty acid processing, including S100A8 and S100A9 (Kragballe and Voorhees, 1987; Fogh *et al*, 1989; Siegenthaler *et al*, 1997).

Kinase regulation of S100A7 and EFABP distribution In addition to calcium, we also assessed the effect of other keratinocyte differentiating agents on S100A7 and EFABP distribution. Interestingly, redistribution of S100A7 and EFABP to peripheral structures was observed when keratinocytes were treated with okadaic acid but not with 12-*O*-tetradecanoyl-phorbol-13-acetate. This suggests that translocation of S100A7 and EFABP is dependent upon phosphatase/kinase activity, but that the kinase is not protein kinase C. The okadaic acid results suggest that translocation may be modulated by protein phosphatase 1 and 2A. Alternatively, the process may be controlled by a kinase that is modified by these phosphatases. In agreement with this suggestion, previous studies show that S100A8 and S100A9 translocation in activated neutrophils and

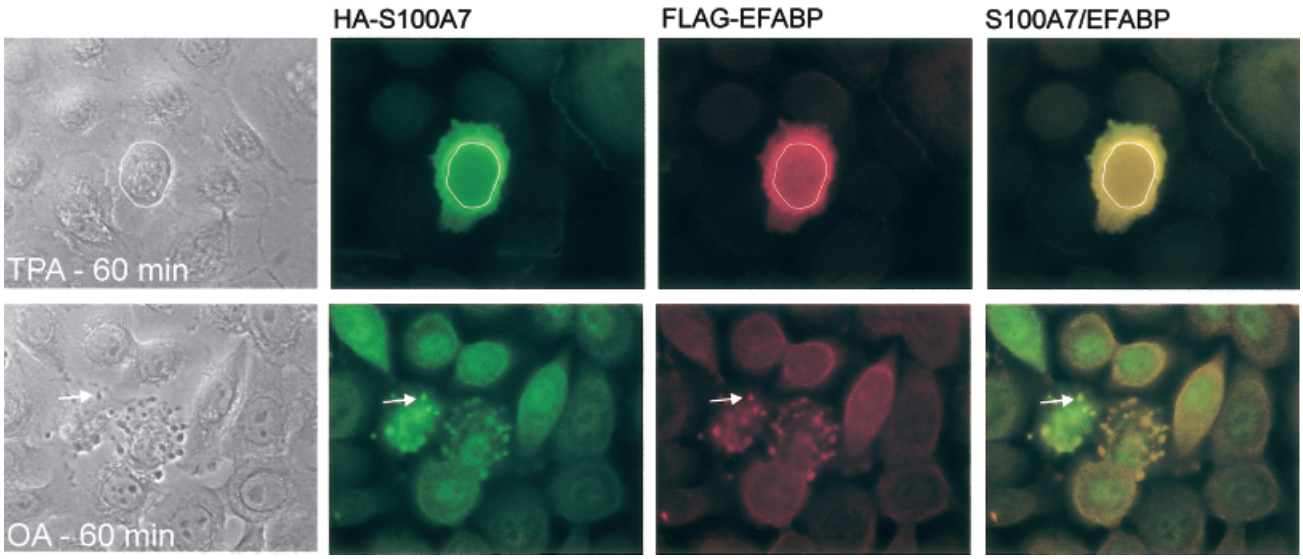

Figure 10. Effect of kinase activators and phosphatase inhibitors on HA-S100A7 and FLAG-EFABP distribution. Keratinocytes were infected with 15 MOI Ad5-HA-S100A7 and 10 MOI of Ad5-FLAG-EFABP. At 48 h postinfection, keratinocytes were treated with 50 ng per mL 12-*O*-tetradecanoyl-phorbol-13-acetate or 100 nM okadaic acid (okadaic acid) for 60 min. The cells were then fixed and incubated with either fluorescein isothiocyanate-conjugated rat anti-HA (green) or Cy3-conjugated mouse anti-FLAG (red). The cells were then photographed using a fluorescence upright microscope. The S100A7/EFABP column shows the combined green and red images—yellow indicates colocalization. The ring in the *top panels* marks the perimeter of the nucleus. The arrows in the *lower panels* mark the peripheral structures containing HA-S100A7 and FLAG-EFABP.

monocytes requires extracellular calcium, and that it may be phosphorylation dependent (Guignard *et al*, 1996; van den *et al*, 1996). Moreover, it has been suggested that a kinase other than protein kinase C might be involved in the calcium-activated signaling that leads to S100A8 and S100A9 translocation (Guignard *et al*, 1996). Additional studies will be required to identify these regulators.

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